

## **1- Cover Page**

### **Proposal to:**

California Department of Food and Agriculture  
Pest Exclusion Branch/Nursery, Seed and Cotton program  
Attn: Katherine Filippini  
1220 N Street  
Sacramento, CA 95814

**Submitting Organization:** University of California Riverside

**Project Title:** Managing Trunk Diseases in Plant Nursery Stock.  
#20-1062-000-SA

**Project Period:** 07/01/20 to 06/30/2023

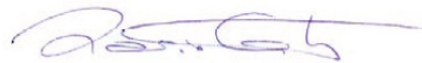
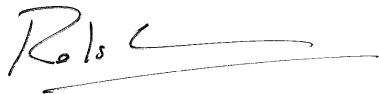
**Project Fiscal Year:** Ongoing, year 3 of 3.

**Amount Requested:** \$139,310

### **Principal investigators:**

Dr. Philippe Rolshausen  
Cooperative Extension Specialist  
Dept. of Botany and Plant Sciences  
UC Riverside  
900 University Ave  
Riverside, CA 92521  
philrols@ucr.edu – (951) 827-6988

Dr. Dario Cantu  
Professor  
Dept. of Viticulture and Enology,  
UC Davis  
146 RMI North Building  
Davis, CA 95616  
dacantu@ucdavis.edu – (530) 752-2929



**Cooperating Personnel:** None

**Checks Made Payable to:** The Regents of the university of California

### **Send Check to:**

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Main Cashier's Office  
900 University Avenue  
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### **Send Award Notice to:**

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## **2- Proposal Narrative**

### **IAB Research Priorities**

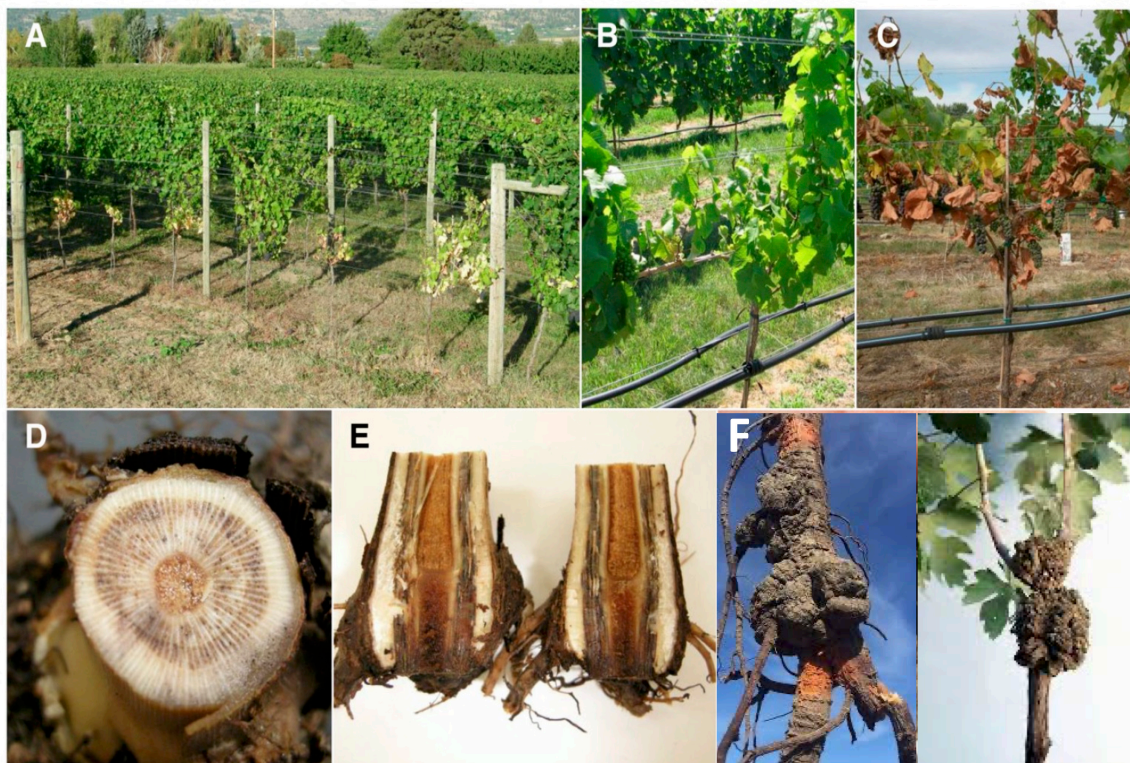
The project addresses the following priority: research on important diseases (fungal trunk diseases and crown gall) that significantly affect the quality of nursery stock. This project will also address education and outreach with the focus on promoting California-produced nursery stock.

### **Project Summary**

The overarching goal of this project is to increase the quality of plant nursery stock by reducing pathogen load. The scope of the work will focus on diseases that affect the plant vascular system including the complex of fungal pathogens causing grapevine trunk diseases and *Allorhizobium vitis* the causal agent of crown gall. We aim to identify, using a combination of traditional microbial techniques and metagenomic sequencing approach, the critical steps during the propagation phase that are conducive to the spread of these pathogens. The outcome of this work will include implementing a comprehensive set of guidelines to reduce the incidence of pathogens in plant stock below acceptable levels and developing accurate, quick and sensitive diagnostic tools.

### **Project's Benefit to Nursery Industry**

Shaping vineyard health starts at the source, in nurseries. Planting healthy vines is the foundation to vineyard longevity and profitability (Baumgartner et al., 2019; Gispert et al., 2020). The presence of vascular pathogens in the planting material of newly established vineyards has been recognized as a cause of poor plant vigor, decreased vine lifespan with reduction of cumulative yield, and a negative impact on income and return on capital (**Figure 1**; Gramaje and Armengol, 2011; Gramaje et al., 2018; Latoya Johnson et al., 2016; Waite and May, 2005). The plant vascular system is the vehicle for water and nutrient transportation and signaling routes between the below (i.e., root) and above (i.e., leaf, fruit) ground plant parts. Fungal pathogens causing grapevine trunk disease (GTD) and *Allorhizobium vitis* (formerly *Agrobacterium vitis* or *A. tumefaciens* biovar 3) the causal agent of crown gall (CG) compromise the physiological functions of the host vascular system thereby limiting plant capacity to uptake and translocate nutrients and water, which reduce vigor and productivity and leads to vine early death. In nursery, sources of infection come either from already infected mother vines or by contamination of clean plant material during the propagation process (Gramaje and Armengol, 2011; Gramaje et al., 2018; Voegel and Nelson, 2018). Wounds are the major point of entry of pathogen inoculum. Because wounds are made at every stage of production from collection and disbudding of cuttings to bench grafting and lifting and trimming of finished vines/trees, chances of pathogen infections are high.



**Figure 1:** Trunk diseases caused by vascular pathogens. Poor vigor and decline of vines affected by esca (A and C) and black foot (B) diseases. Wood necrotic lesions in rootstocks affected by esca (D and E) and gall symptoms on crown and cane(F).

Certification programs give insurance that commercial plants sold by nurseries are free of pathogens. The California Grapevine Registration & Certification (R&C) Program and 2010 Protocols standards have been instrumental for providing grape growers plants free of several pathogens (several viruses, phytoplasma, Xylella; <https://fps.ucdavis.edu/fgr2010.cfm>; Fuller et al., 2019). Viruses are commonly host-specific or have a narrow host range and can be eliminated by shoot tip and meristem culture. In contrast, there is no certification program for pathogens causing GTD and CG for obvious reasons, even if those have been clearly identified in nursery plant propagation pipelines. First, GTD is caused by a complex of several fungal taxa with broad host and environmental range, making it impossible to eradicate all of them from the propagation pipeline. Second, fungi causing GTD can be natural endophytes and can live in association with the plant without causing disease and thus can go undetected because plant are asymptomatic. However, under plant stress conditions often met during the propagation phase (heat stress, wounding), these saprotrophic fungi can become pathogenic. *Allorhizobium vitis* is a systemic bacterium in grapevine and has been detected in lignified canes, green shoots and xylem sap (Latoya Johnson et al., 2016). It has also been shown that the pathogenic bacterium is capable of colonizing shoot tips and meristems (Latoya Johnson et al., 2016), making it very difficult to eliminate this bacterium from the propagation pipeline.

Nurserymen are aware of the impact of grapevine trunk disease and crown gall on the quality of plant nursery stocks. The majority of California nurseries have implemented best practices to maintain high quality cuttings and minimize chances of pest and pathogen infections that include maintaining clean mother blocks, sanitation of bench grafting and disbudding machines, hot water baths, as well as treatments of plant materials with bleach, ozone, conventional fungicides and biological agents (*Trichoderma* and *Mycorrhizae*). However, despite those efforts, GTD and CG have remained a concern for the nursery industry, and it has been looking for practical cost-effective solutions to address the problem. One of the major issues possibly stem from the use of hot water baths implemented to eradicate pests, but the water temperature and bathing period may not be optimal to kill fungal and bacterial pathogens associated with a minority of asymptomatic vines and could result in a high rate of infection of clean plants (Mahmoodzadeh et al., 2003.; Waite et al., 2015).

This proposal is **not aimed at implementing a certification program for pathogens causing GTD and CG**, because it would be unrealistic and too costly. However, the nursery industry must be proactive at developing quality control measures to maintain infection under acceptance levels and extend the scientific information to grape growers so that a zero tolerance for the causal agents of CTG and CG don't become the new benchmark.

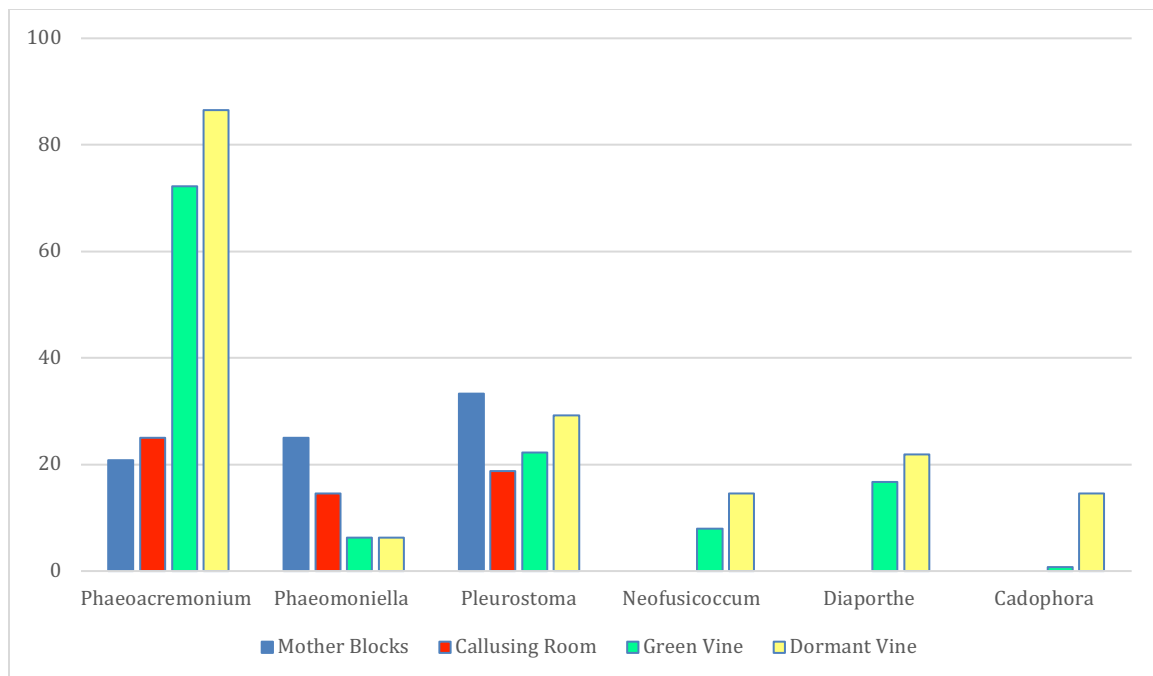
### **Objectives**

- 1-** Identify fungal trunk disease infection routes in nursery.
- 2-** Profile trunk disease pathogens in nursery stock to improve accuracy of diagnostic tools.
- 3-** Provide industry guidelines and training for best management practices and disease diagnostic.

### **Workplans and Methods**

**Objective 1.** Identify fungal trunk disease infection routes in nursery.

We have established a partnership with several nurseries that are members of the California Fruit Tree, Nut Tree, and Grapevine Improvement Advisory Board. In the first two years, we have sampled a total of 825 vines at four different steps of the plant propagation pipeline, that include the mother blocks, the callusing room, as well as green and dormant vines. We deployed standard culture dependent diagnosis (Rolshausen et al., 2013) and culture-independent molecular approaches (Morales-Cruz et al. 2018) to profile fungal communities and pathogen load. Our initial results confirmed that many fungal pathogens were present in nursery stocks. Results suggested several potential different infection routes with endogenous pathogens (*Phaeoacremonium*, *Phaeomoniella*, *Pleurostoma*) being present in mother vines and spread during the propagation phase, whereas exogenous infection from airborne and soilborne pathogens (*Neofusicoccum*, *Diaporthe*, *Cadophora*) occurred later, in dormant and green vines (**Figure 2**).



**Figure 2:** Percent incidence ([number of vines infected/the total number of vines tested] x 100) of the most common fungi causing grapevine trunk disease in plant nursery stocks at different stages of the propagation.

In the third year we intend to expand the sampling to additional nurseries that recently joined our research efforts and repeat sampling vines for a second year from nurseries that were already participating in the project. The focus of our sampling is on the same plant genetic materials across nurseries to alleviate a plant genetic effect. Our sampling design is to collect 25 vines of the popular clones Chardonnay Clone 4 x 1103P rootstock and Cabernet Sauvignon FPS 8 x 1103P rootstock at each of the four stages of the propagation pipeline. For the standard diagnostic with culture dependent approach, cuttings are split in half and the wood health is rated (see IAB 2021 Progress Report for more information) and wood tissue is plated on culture medium from three areas of the cuttings that include above the graft union (scion), below the graft union (rootstock), and at the root ball (rootstock). Fungi recovered are identified taxonomically based on DNA sequencing (Rolshausen et al., 2013). This information will be useful to build the database of the most common fungal species found in California nurseries and develop diagnostic tools (Objective 2). For the culture-independent approach, total DNA is extracted from composite of five wood samples from each area of the cutting and fungal trunk pathogens are profiled using the specific, sensitive and accurate metabarcoding ITS primers described by Morales-Cruz et al. (2018).

This year, we will also include environmental samples from the water baths, potting soil, and peat moss to determine if fungal pathogens can be identified in those substrates. We will only use a culture independent approach that will consist of extracting total DNA from these environmental samples and profile fungi using standard DNA metabarcoding methods (Deyett et al., 2020; Morales-Cruz et al.,

2018). In addition, we will expand our plant diagnostic to *Allorhizobium vitis* and testing the total DNA samples already obtained from the vine cuttings for fungal community profiling. We will also test calli collected from cuttings at the callusing stage. A quantitative PCR (Eastwell et al., 1995) and digital droplet PCR (Voegel and Nelson, 2018), will be tested for *A. vitis* diagnosis. Both assays will use primers that target the virulence gene (VirA) located on the Ti plasmid of *A. vitis* due to its essential role in pathogenesis and utilize the SYBR Green fluorescent dye for target DNA quantification.

**Objective 2.** Profile trunk disease pathogens in nursery stock to improve accuracy of diagnostic tools.

The information collected in objective 1 provide the building blocks for a database of the fungal trunk pathogens occurring in California nursery. Providing a list of the most common trunk pathogens species will help with the design of specific, sensitive, and reliable diagnostic tools that will be helpful for plant quality control purposes and decision-making.

**Table 1:** Percent Incidence of fungal pathogens ([number of vines infected/the total number of vines tested] x 100) in plant nursery stock (n=400 vines).

Pathogen Name	Disease Associated with Pathogen	Percent Incidence
Phaeoacremonium	Esca, Young Vine Decline, Measles	65.3
Pleurostoma	Esca, Young Vine Decline, Measles	24.3
Diaporthe	Phomopsis canker, cane and leaf spot	14.4
Phaeomoniella	Esca, Young Vine Decline, Measles	8.7
Neofusicoccum	Bot canker	8.1
Cadophora	Esca, Young Vine Decline, Measles	4.7
Campylocarpon	Black foot	1
Neoscytalidium	Bot canker	0.8
Eutypa	Eutypa dieback	0.6
Phialophora	Esca, Young Vine Decline, Measles	0.4

In the initial two years of our survey, we identified several soilborne fungi (*Fusarium*, *Rhizoctonia*) known to be pathogenic in many plant systems. We are now completing Koch's postulate using standard methods (Rolshausen et al., 2013) to determine pathogenicity of those fungi to grapevine. *Fusarium* and *Rhizoctonia* isolates recovered from nursery cuttings were inoculated on grapevine cuttings in

2022 by drilling a hole in the cuttings and filling it with an agar plug with fungal mycelium. Grapevines will be incubated for one full year in the greenhouse at UC Riverside and in 2023 grapevine will be rated for disease. Necrotic lesions length will be recorded and compared to control plants inoculated with blank agar plugs and fungal isolates will be recovered from necrotic lesions to confirm that symptoms are caused by the fungal isolates. If both symptoms development and fungal isolations are confirmed, those fungi will be included in our database.

Once we have collected a significant number of grapevine samples across several nurseries and built a robust fungal database our team will develop PCR diagnostic tools. We will design specific primers that target the main fungal pathogenic species identified at the different stages of the plant propagation. PCR protocols will be optimized and made publicly available so that private diagnostic labs can routinely use them to test nursery samples. Training will also be provided to nursery that have the capacity to house their own diagnostic lab.

**Objective 3.** Provide industry guidelines and training for best management practices and disease diagnostic.

Our outreach and extension efforts will be multi-faceted. The deliverables will include a general set of guidelines that are applicable to all nurseries to reduce infection threshold of pathogens causing GTD and CG under acceptable levels. Those guidelines will be developed in the form of printed pamphlet or available online in both English and Spanish and will educate nurserymen about adopting the best practices to maintain clean nursery stock. In addition, we will provide recommendations that are tailored to individual nursery, as each nursery have implemented their own specific practices. Another deliverable will include training of nursery for GTD and CG disease diagnostic. Finally, we hope to develop in partnership with nurseries an article that could be published in industry newsletter and perhaps a video that can be made available online, that promotes California-nursery stock and communicates to grape industry stakeholders the practices adopted by California nurseries to maintain clean stocks.

### **Project Management and Evaluation**

PI Rolshausen has been working directly with nurseries and visiting them on a regular basis to address all three objectives. Rolshausen has been collecting sample materials (vines, soil, or water) and bringing samples back to UC Riverside where it is processed in the laboratory for culture-based diagnostic. Frozen samples have been shipped to PI Cantu at UC Davis where it is further processed for culture-independent diagnostic. Results from the Cantu and Rolshausen labs have been summarized in the form of annual reports to the funding agency. Nurseries collaborating on the project have been kept updated of the research progress on a regular basis through phone calls and emails. All results have been kept confidential.

### **Literature Review**

Management of fungal trunk diseases in nurseries has been an important research topic especially for production of commercial grape stock in major viticulture regions worldwide including Europe, South Africa and New Zealand. In response to this threat, the European Cooperation in Science and Technology (COST) Action FA1303 “Sustainable Control of Grapevine Trunk Diseases” (funded by the European Union), was initiated in 2013 (Gramaje and Di Marco, 2015). The main aim of this initiative was to develop a network of European expertise to improve the understanding of grapevine trunk diseases (GTDs), by acquiring knowledge of pathogen epidemiology, vine/pathogen interactions and the ecology of wood-inhabiting microorganisms. One of the outcomes was to recommend protocols that prevent and reduce the impacts of pre-existing GTD pathogen infections in nurseries because there is clear evidence that plants become compromised because of the multiple wounding injuries combined with stress factors during the propagation of plants (Waite et al., 2018). Up until now with this current funded project, there has been little research done on this topic in California nurseries. Most of the research and extension on fungal trunk diseases has been focused on the field production aspects, including understanding the etiology of those disease, their economic impact and developing management strategies (Baumgartner et al., 2019; Kaplan et al., 2016; Rolshausen et al., 2010). Our team has developed a rapid and cost-effective method based on Next Generation Sequencing (NGS) technologies for the detection, identification and quantification of trunk pathogens in grapevine wood that can be applied to nurseries (Morales-Cruz et al., 2017; Morales-Cruz et al., 2018). We have demonstrated that NGS detection is quantitative and allows for a quick and reliable detection of latent pathogens.

*Allorhizobium vitis* is not on the list of pathogens for the ‘Protocol 2010’ and as such are not being tested by nurseries, although vines showing gall-like symptoms are removed from the field. *Allorhizobium vitis* is often introduced to vineyards through infected plant nursery material and can reside in the soil overtime (Voegel and Nelson, 2018). Mechanical or freezing wounds suffered by the grapevine often result in new infections. Crown gall is especially a problem in cold viticulture areas experiencing winter freezing damage. In nursery, *A. vitis* enters plants through grafting wounds and systemically colonize the host, including the shoot tip meristem, making it challenging to eradicate it with shoot tip and meristem cultures (Latoya Johnson et al., 2016). In addition, thermotherapy indicated mixed results with respect to eradicating the pathogen, depending on the water temperature and water bath time length (Mahmoodzadeh et al., 2003). *Allorhizobium vitis* carry a Ti (Tumor-inducing) plasmid that causes crown gall by transferring the T-DNA region of the tumor-inducing bacterial plasmid to the host cell, which subsequently integrates into the plant host genome. The inserted T-DNA contains genes for biosynthesis of plant growth hormones which eventually leads to abnormal gall formation as well as genes for biosynthesis of opines, which serve as nutrients for the bacterium (Burr and Otten, 1999). Research has focused on pathogen diagnosis (Eastwell et al., 1995; Voegel and Nelson, 2018) and control methods using avirulent *A. vitis* that does not carry a Ti plasmid (Kawaguchi and Inoue, 2012).

## References Cited.

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### **Current and Pending Sources of Support for this project**

The third year of this CDFA-IAB project builds on a long-term research program led by Drs. Cantu and Rolshausen. Our group has already provided comprehensive genomic information on fungi associated with trunk diseases and developed new sensitive sequencing-based tools that can accurately detect and identify the causal agents. Using those tools and in partnership with California nurseries, we showed that propagation plant materials contain latent fungal infections. We submitted this year a new proposal entitled 'Assessment of Plant Nursery Stock Quality and its Impact on Vineyard Health' to the American Vineyard Foundation, the California Grape Rootstock Improvement Commission and California Grape Rootstock Research Foundation consortium, that is directly in line with the IAB proposal because it bridges management of trunk diseases from the nursery to the vineyard.

**EXHIBIT '1B'**  
**IAB - BUDGET PROPOSAL**

**Project Title/Description:** Managing Trunk Diseases in Plant Nursery Stock

**Project Leader:** Philippe Rolshausen, University of California Riverside

**Proposed Fiscal Year:** 2022-2023

**A. PERSONNEL SERVICES:**

Graduate Student Researcher @ \$2,589/mo. for 12 mo.	\$31,072
Graduate Student Researcher Tuition & Fees	\$16,534
Graduate Student Researcher Benefits = 1.80%	\$559
<b>TOTAL PERSONNEL SERVICES</b>	<b>\$ 48,165</b>

**B. OPERATING EXPENSES:**

Laboratory Supplies	\$5,000
Travel (per diem)	\$3,000
Postage	\$0
Other: Sub-Award to UC Davis, Co-PI Dario Cantu (See Attachment 2B for Sub Award budget detail)	\$78,328
<b>TOTAL OPERATING EXPENSES:</b>	<b>\$134,493</b>

**C. INDIRECT COST:**

**\$4,817**

**D. TOTAL BUDGET REQUESTED:**

**\$139,310**

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**EXHIBIT '2B'**  
**IAB - SUBAWARDEE BUDGET PROPOSAL**

**Project Title/Description:** Managing Trunk Diseases in Plant Nursery Stock

**Project Leader:** Dario Cantu, University of California Davis

**Proposed Fiscal Year:** 2022-2023

**A. PERSONNEL SERVICES:**

Graduate Student Researcher @ \$2,600/mo. for 12 mo.	\$31,208
Graduate Student Researcher Tuition & Fees	\$33,585
Graduate Student Researcher Benefits = 2.20%	\$687
<b>TOTAL PERSONNEL SERVICES</b>	<b>\$65,480</b>

**B. OPERATING EXPENSES:**

Laboratory Supplies	\$300
Travel (per diem)	\$0
Other: DNA Sequencing	\$6,000
<b>TOTAL OPERATING EXPENSES:</b>	<b>\$71,780</b>

**C. INDIRECT COST:** **\$6,548**

**D. TOTAL BUDGET REQUESTED:** **\$78,328**

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